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13. ABSTRACT ***(Maximum 200 Words)***

By the time a cancer is detected, its tumor cells already exhibit myriad genetic abnormalities. To gain a better understanding of genetic events that occur early in breast carcinogenesis, this research examined genetic abnormalities 1) in histologically normal tissue from women at low, medium or high degrees of breast cancer risk, using archival specimens of reduction mammoplasties, and of diagnoses of atypical hyperplasia and breast cancers, respectively; and 2) in synchronously occurring putative precursor lesions, including normal-appearing epithelium, simple and atypical proliferative (hyperplastic) lesions and carcinomas themselves.

Each specimen is microdissected, its DNA examined using a panel of selected microsatellite markers, and evidence of clonal abnormalities sought, in particular loss of heterozygosity (LOH) and microsatellite instability (MI).

Investigation of the project's first goal generated data regarding the timing and sites of early genetic abnormalities. These data raise the possibility that a field defect exists in certain breast tissue. Investigation of the second goal is uncovering that a variety of clonal relationships exist between multiple synchronous putative precursors. These studies are identifying important sites of genetic abnormalities in early breast cancer precursors, and begin to outline a sequence of acquired genetic abnormalities needed for precursor lesions to evolve into full-blown malignancies.

14. SUBJECT TERMS
Proliferative, field defect, microsatellite, clonality, loss of Heterozygosity, breast cancer

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Table of Contents

Introduction:

Subject, purpose and scope of research:

By the time carcinomas *in situ*, regarded as the earliest human breast cancers, are detected, tumor cells already exhibit multiple recurrent genetic abnormalities. This implies that earlier precursor lesions must exist, and that these precursors likely contain genetic abnormalities critical to the early stages of tumor development. The goal of this research has been to gain a better understanding of the identity of these precursor lesions, and of the genetic abnormalities they contain. Therefore, the research project's two objectives were 1) to determine whether histologically normal breast ducts contain genetically abnormal monoclonal populations of cells by determining whether DNA abnormalities were detectable in histologically normal tissue from women at three different levels of risk for breast cancer (those undergoing reduction mammoplasty (RM); those diagnosed with high-risk proliferative lesions, i.e., atypical hyperplasia (AH); and those diagnosed with breast cancer); and 2) to investigate the clonal evolution of proliferative lesions by examining the DNA "fingerprint" of multiple synchronous breast tissue samples: normal-appearing epithelial ducts and terminal ductal/lobular units (TDLUs), simple and atypical hyperplastic lesions and malignant tissue from a series of breast cancer specimens. These studies were designed to help elucidate some of the earliest abnormalities in human breast carcinogenesis.

Body:

Research accomplishments associated with each task outlined in the approved Statement of Work:

Task 1. Months 1-8: Selection of microsatellite primers to be tested, testing combinations for multiplex reactions, choosing final primer combinations.

Outcome: This task has been completed. Multiple primers were tested, a panel of 9 were used for Technical Objective 1, an optimized and expanded panel of 18 were used for Technical Objective 2. The panel used for Objective 1 is included in the paper reporting the results (Larson et al, Genetically abnormal clones in histologically normal breast tissue. Amer J Pathol 1998; 152:1591) (see attached). The panel used for Objective 2 is shown below.

Table 1: Expanded Marker Panel (*n*=18): Chromosomal Sites and Type of Repeat

Chromosomal Site	Marker	Repeat Type
1q32-42	D1s549	tetra
	D1s213	di
3p24	D3s1283	di
7q31	D7s486	di
11p15	THO1	tetra
	D11s2071	di
11q13	PYGM	di
11q23	D11s1818	di
	D11s1819	di
16q22-24	D16s402	di
	D16s413	di
	D16s512	di
17p13.1	TP53	di
	D17s796	di
	D17s525	di
17q21	D17s1290	di
	D17s579	di
Xq11-12	AR	tri

Task 2. Months 1- 8: Identification of specimens. Specimens that belong to the three categories of subjects whose "normal" ducts will be studied will be identified from pathology reports stored in the pathology department archives, specimens for which

blocks are not available will be eliminated. Specimens of breast cancer will be identified and reviewed with the pathologist to identify geographically discrete AH lesions.

Outcome: This task has been completed. Acquisition of the 20 specimens analyzed in Objective 1 was achieved during months 1-8. Acquisition of the 18 specimens analyzed in Objective 2 was also achieved, but was delayed into years 2 and 3 of the award because genetic analysis of these specimens has taken slightly longer than anticipated.

Task 3. Months 3-22: Section acquisition. Review existing slides from all potential specimens with pathologist to confirm histologic diagnoses, if confirmed, have serial sections cut, stain and review again with pathologist, who will assist in marking lesions to be microdissected. Repeat until each subject has an adequate number of ducts/lobules ready for microdissection.

Outcome: All sections required for genetic analyses proposed in Objective 1 were acquired during the proposed time. All sections required for genetic analyses proposed in Objective 2 also have been acquired. As indicated above, the genetic analyses proposed in Objective 2 have taken somewhat longer than expected. Because DNA can degrade over time, it was preferable to acquire sections just before we were ready to analyze them. Therefore, we acquired sections for Objective 2 throughout Year 3 of the award. Some of these remain to be analyzed: this work will be performed during the 1 year no-cost extension of the award that has been requested.

Task 4. Months 4-28: Microdissection and DNA extraction. Perform individual microdissections of demarcated tissue. Extract DNA from each microdissected area.

Outcome: All microdissections and DNA extractions needed for Objective 1 have been performed. Results have been analyzed, and reported (Larson et al, Genetically abnormal clones in histologically normal breast tissue. Amer J Pathol 1998; 152:1591) (see attached). Microdissections and DNA extractions have been performed for 16/18 cases being investigated in Objective 2. Two of 18 cases remain to be completed, this work will be performed during the 1 year no-cost extension of the award that has been requested.

Task 5. Months 6-32: PCR. Perform multiplex PCR on DNA from each microdissected area. Repeat all abnormal or indeterminate reactions.

Outcome: All multiplex PCRs for Objective 1's experiments have been completed, and reported (Larson et al, Genetically abnormal clones in histologically normal breast tissue. Amer J Pathol 1998; 152:1591) (see attached). All multiplex PCRs on 16/18 cases to be studied in Objective #2 have been performed. Two remaining cases remain to be analyzed; this work will be performed during the 1 year no-cost extension of the award that has been requested.

Task 6. Months 9-34: Analyze data. Review all gels, tabulate number and type of microsatellite abnormalities, enter data into databank. For Technical objective #2, determine if multiple patterns of monoclonal microsatellite alterations are seen in the premalignant lesions surrounding breast cancers, and if any share the microsatellite "fingerprint" of the cancer itself.

Outcome: Data Analysis of Objective #1 has been completed. The analysis of the data generated indicates that differences exist in the rates of clonal microsatellite abnormalites in histologically normal epithelium among women at no increased risk of breast cancer (those undergoing reduction mammoplasty [RM]) vs. those at increased risk, i.e., diagnosed with the high-risk proliferative lesion atypical hyperplasia (AH) vs. those at highest risk, i.e., diagnosed with breast cancer itself. Analysis comparing all three groups and using the 2-sided Fisher's exist test, which is most appropriate given the small sample size, yield a p value of 0.107, which is suggestive given sample size. Using the same statistical tests, analysis of women <50 years [since age could be an unrecognized factor contributing to the rate of genetic abnormalities], and comparing RM vs breast cancer cases, yields a p value of 0.049.

In addition, we reexamined the likelihood that the distribution of abnormalities we found could be due to chance. We noted 28/35 (80%) abnormalities were at 4 markers located at sites believed important in breast cancer development: 7q31, 11p15, 17p13, 17q21. This result is not likely due to chance, as the increased occurrence of these abnormalities at these sites in comparison with the other 5 sites is statistically significant ($p < 0.01$) (Fisher's exact test). Although these 4 markers were selected because of their chromosomal location, their overrepresentation among all abnormalities indicates that mutations near these sites may predispose to the formation of genetically aberrant clonal populations. In contrast, mutation at arbitrary or neutral sites may not confer a growth advantage, and a detectable mutant clone may not arise. This suggests that the genetic alterations detected are less likely to be random changes and more likely to be relevant to the earliest stages of breast cancer development.

Representative examples of the autoradiography and some of the statistical analyses have been published (Larson et al, Genetically abnormal clones in histologically normal breast tissue. Amer J Pathol 1998; 152:1591); others were reported in Annual Report #2. We believe that these results are suggestive but only preliminary. For more definitive analyses, we look forward to accumulating additional subjects and thereby improving the confidence in our results. We plan to accumulate some additional subjects during the 1-year no cost extension that has been requested.

Data Analysis of Objective #2 is ongoing. Substantial progress has been made and a manuscript is in preparation. Work on this aim is of great importance because although Objective #1 has demonstrated that normal-appearing epithelium can be genetically abnormal, the meaning of these abnormal clones is uncertain. They may represent

precursor lesions, or clonal "dead-ends". We aim to investigate ~ 18 cases. Thus far, from 16 independent cases, we have analyzed a total of 222 distinct lesions. These lesions' histologies are depicted in Table 2:

Table 2: Microdissected lesions: number and histology

Case:	No. lesions per histology					
	S, L	*N*	*H*	*AH*	*CIS*	*INV*
2004	-	5	1	3	3	-
2008	-	4	-	1	2	2
2012	-	4	-	4	-	2
2014	1	1	-	1	1	-
2028	3	7	-	1	1	-
2031	2	5	-	1	3	2
2032	3	6	2	-	-	2
2034	-	9	-	1	1	-
0038	-	5	-	3	3	5
0039	-	4	2	8	4	-
0052	1	8	1	2	2	3
0053	1	9	2	2	2	1
0070	-	4	-	5	3	-
0071	-	9	3	6	2	2
0072	1	10	1	2	3	1
0074	-	11	1	4	2	-
Total	12	101	13	44	32	20

Each of these 222 samples has been analyzed with the marker panel. We find that the proportion of cases, and of lesions, that are clonal, increases with increasingly aberrant histology. We also find that fractional allele loss (FAL), a commonly used measure of the amount of chromosomal loss per sample, increases with increasingly aberrant histology. Finally, we find that extent of loss of chromosomal material (loss of heterozygosity, LOH) changes with increasingly aberrant histology. Single loci tend to be lost in the

normal and simple hyperplastic lesions, suggesting that mitotic recombination might be the mechanism of LOH, whereas all evaluable lesions tend to be lost in the carcinomas. The atypical hyperplasias contain both pattern. These results are summarized in Table 3:

Table 3. Clonality, fractional allele loss (FAL) and extent of LOH by histology.

Samples	**% cases with clones***	**% lesions that are clonal**	**FAL****	**Extent of LOH**
Normal ducts/TDLUs	7/16 (44%)	13/101 (13%)	0.02	Single locus
Hyperplasia (simple)	2/8 (25%)	2/13 (15%)	0.03	Single locus
AH	10/15 (66%)	21/44 (48%)	0.20	Single locus, entire arm
CIS	14/14 (100%)	32/32 (100%)	0.36	Arm >> locus
Inv Cancer	9/9 (100%)	20/20 (100%)	0.42	Arm >> locus

*Clone = LOH at 1 or more sites in a given sample.
**FAL = fractional allele loss

Next, we examined clonal progression of the premalignant lesions. We defined clonal progression or linkage as LOH of the same allele of a marker in tissues of different histologies. We found that clonal linkage to malignancy increases with histologic aberrancy. For instance, only 1/13 (8%) genetically aberrant but histologically normal lesions shared the same LOH as a synchronous cancer. But 15/21(71%) genetically aberrant AH were clonally linked to synchronous malignancies. Table 4 reviews this data.

Table 4. Clonal Progression.

Samples	**Progression to Malignancy**	
	# cases with clones	*# clonal lesions*
"Normal" tissue	1/7 (14%)	1/13 (8%)
Hyperplasia (simple)	1/2 (50%)	1/2 (50%)
AH	8/10 (80%)	15/21 (71%)

Figure 1, below, shows representative examples of the data indicating that genetically aberrant clones in normal-appearing breast epithelium are often not linked to synchronous cancers.

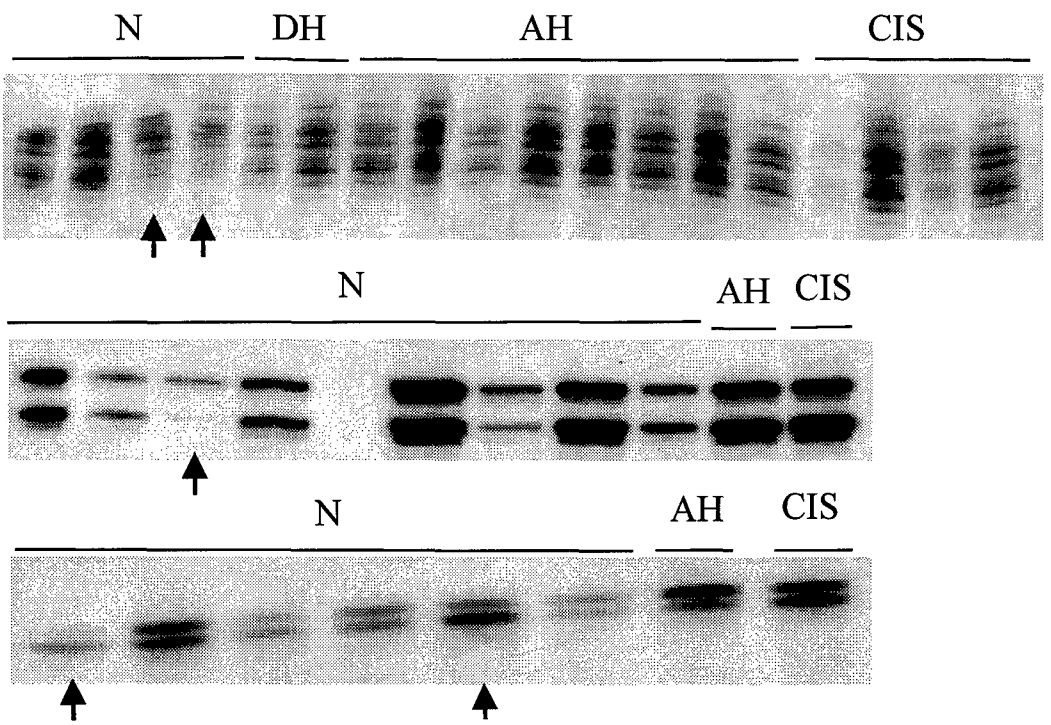

Figure 1: Aberrant clones in normal-appearing breast epithelium can be distinct from synchronous malignant clones. Top panel: LOH (arrows) at marker 17s579 is seen in 2 normal samples, but at no other lesions, in case 0039. Middle panel: LOH (arrow) seen at marker 1s549 in a normal duct, but no other samples, in case 2034. Bottom panel: LOH (arrows) of larger allele at marker THO1 in 2 normal ducts, but no other lesions, in case 2034.

Figure 2, below, shows proliferative lesions, particularly AH, are clonally linked to synchronous malignancies.

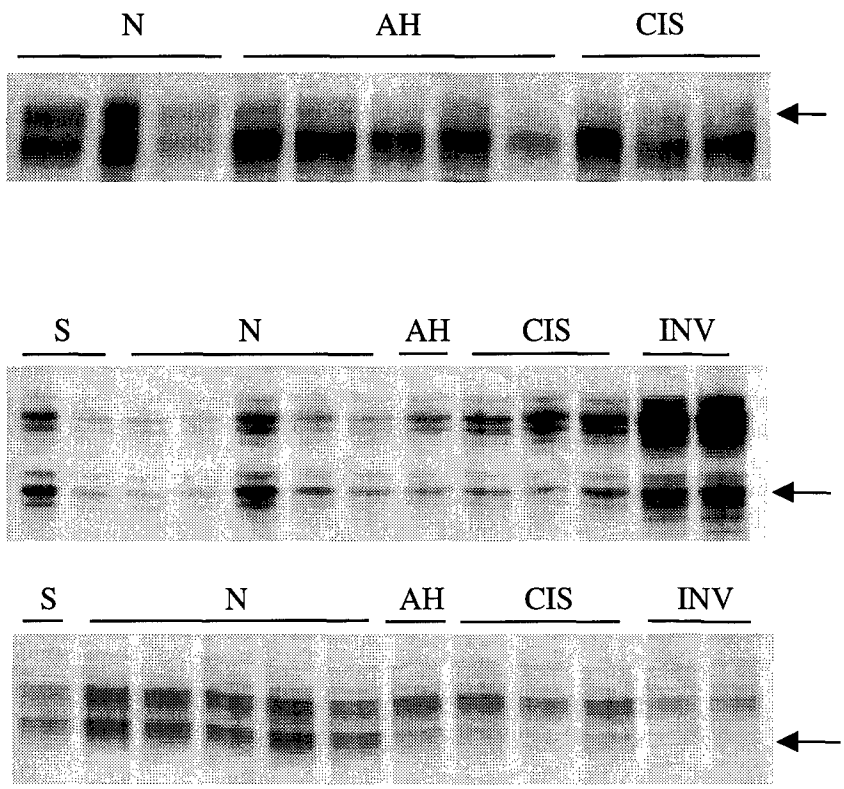

Figure 2: Atypical hyperplastic lesions are clonally linked to synchronous cancers. Top panel: LOH of the larger allele at marker 413 is seen in all examples of AH and of CIS. Middle panel: LOH of the smaller allele at marker 213 is seen starting in the AH lesion and with greater intensity in CIS and INV samples. Bottom panel, same subject, also showing LOH of the smaller allele of marker PYGM starting in the AH lesion and with greater intensity in CIS and INV samples. Arrows indicate the allele that undergoes LOH.

Nevertheless, we find that the most common sites of LOH are the same in normal-appearing epithelium, proliferative and malignant tissue (see Table 5, below):

Table 5: % LOH on chromosomal arms by histology

Arm	% LOH		
	Normal	*Hyperplasia*	*Cancer*
1q	20	37	63
3p	0	10	20
7q	11	0	11
11p	15	15	29
11q	3	39	53
16q	27	57	74
17p	0	21	70
17q	15	0	42
Xq	0	0	0

These data suggest that these common sites of LOH identify the location of candidate early acting tumor-suppressor genes. We speculate that some sort of genomic instability must exist early in the evolution of breast cancer, and leads to loss of chromsosomal material. Some of the material that is lost may contain these candidate tumor suppressors whose inactivation permits clonal expansion.

Task 7. Months 12 and 24: Statistical analysis. For Technical Objective #1, preliminary statistical analysis will be performed to determine rate of microsatellite abnormalities, differences between groups, significance and power of the findings. The preliminary analysis may indicate a need for additional samples from one or another of the subject groups in order to achieve statistically meaningful results.

Outcome: The statistical analyses have been presented above (see Task 6). These analyses indicate significant differences between the rate of microsatellite abnormalities in normal appearing breast epithelium in the controls (RM) vs the breast cancer group. Consistent with these findings, subjects diagnosed with AH (i.e., those at intermediate risk) had an intermediate rate of microsatellite abnormalities. Therefore, a new research grant has been submitted to the NIH to confirm these differences and examine them in greater detail and utilizing additional groups of subjects (i.e., those with constitutional mutation of BRCA1 or BRCA2).

Task 8. Months 30-36. Clinical correlation and follow-up. Review medical records to determine if presence of microsatellite alterations in histologically normal breast tissue is correlated with clinical features. Determine if presence of multiple, monoclonal, genetically distinct premalignant lesions is associated with clinical features of the breast cancer.

Outcome: We have reviewed the clinical data available and determined that the presence of clonal microsatellite alterations in normal-appearing breast epithelium is linked to breast cancer risk, particularly in women < 50 yrs (see Task 6, above). No other associations could be found, but the numbers of subjects available for analysis was small. The role that age plays is unclear. Based on the preliminary results from these studies, we have proposed that subjects < 50 and> 50 be analyzed separately.

Task 9: Months 34-36. Final statistical analysis.

Outcome: Because the statistical analyses performed at months 12 and 24 were considered complete, no final statistical analysis was performed. Instead, during these months we have been focussed on tasks pertinent to completing Objective #2.

Key Research Accomplishments:

1. Demonstration that monoclonal microsatellite DNA abnormalities are detectable in histologically normal breast epithelium from women at all degrees of breast cancer risk. The rate of abnormalities increases with increasing risk, especially among women < 50 years ($p < 0.05$).

2. Identification 18 independent cases of archival tissue containing synchronous normal, proliferative and malignant breast tissue; identification and microdissection of 222 discrete lesions from each specimen; DNA extraction and fingerprinting of each lesion; creation of spreadsheet to organize data. Approximately 25 lesions from 2 remaining cases remain to be analyzed.

3. Preliminary analysis confirms fairly high rates of abnormal clones in histologically normal breast tissue from women < 50 years with breast cancer, and suggests that these clones are rarely linked clonally to synchronous cancers. In contrast, high-risk proliferative lesions (atypical hyperplasia) are frequently linked clonally to synchronous cancers. Overall, however, sites of LOH in normal-appearing, hyperplastic and malignant tissue are the same, suggesting that these sites are near regions containing candidate tumor suppressor genes whose inactivation is important early in breast carcinogenesis.

Reportable Outcomes:

Manuscripts:

1. Genetically Abnormal Clones in Histologically Normal Breast Tissue. PS Larson, A de las Morenas, LA Cupples, K Huang, **CL Rosenberg.** Am J Pathol 1998; 152:1591.
2. Clonal Progression of Premalignant Breast Cancer Precursors. PS Larson, A de las Morenas, **CL Rosenberg**. Manuscript in preparation.

Abstracts:

1. Genetically Abnormal Clones in Histologically Normal Breast Tissue. **CL Rosenberg.** Gordon Research Conference: DNA Alterations in Transformed Cells. Colby - Sawyer College, New London NH, 8/11/98.
2. Clonal Progression of Premalignant Breast Cancer Precursors. Larson PS, de las Morenas A, **Rosenberg CL**. Proceedings AACR, 4/2000 #3277.
3. Clonalitiy and Genetic Instability in Premalignant Breast Tissue. **CL Rosenberg**, A de las Morenas, LA Cupples, PS Larson. Department of Defense Breast Cancer Research Program meeting, invited platform presentation in the Molecular Epidemiology program. Atlanta GA, June 8-11 2000.

Funding applied for based on work supported by this award:

1. NIH R01CA81078 (application awarded: funding to begin in 2001).

Conclusions:

We conclude that histologically normal breast epithelium may harbor genetic abnormalities. The rate of abnormalities increases with increasing risk of breast cancer, especially among women < 50 years ($p < 0.05$). This has implications for our understanding of the earliest steps of breast tumorigenesis, which may begin before any pathologic changes are evident. Investigation of more subjects, and subjects from additional risk groups, should confirm and expand these findings.

Further, we conclude that only a small subset of these aberrant clones in normal-appearing tissue are clonally linked to synchronous breast cancers. Thus, multiple aberrant clones can coexist. Their eventual fate cannot be determined, but it is of interest that the same sites of LOH are detected in normal-appearing, proliferative and malignant tissue, suggesting that LOH at these sites may inactivate a tumor suppressor gene and lead to clonal expansion. In contrast to normal-appearing clones, most high-risk atypical hyperplastic lesions are clonally linked to synchronous breast cancera, indicating that these lesions are more likely to be true precursors rather than markers of increased risk.

Appendix:

1. Manuscript (attached)
2. Abstracts (#3)

a) 8/11/98:

Genetically Abnormal Clones in Histologically Normal Breast Tissue

CLRosenberg, Boston University Medical Center, Boston MA 02118.

Breast cancer is a genetic disease, but little is known about the genetic abnormalities that are central to the earliest steps of tumorigenesis. Identifying these abnormalities may be critical to understand breast cancer risk and development, and to create new detection and treatment strategies. To elucidate what these important early abnormalities might be, we have developed a system to investigate small quantities of archival human breast tissue specimens. This system utilizes ~20 highly heterozygous microsatellite markers (mono, di, tri and tetranucleotide repeats), located at ~11 chromosomal regions (including some potentially relevant to breast tumorigenesis), multiplexed into ~5 PCRs. With these combinations we can reliably examine nanogram quantities of DNA from microdissected tissue sections.

Using this system, we found monoclonal, genetically abnormal populations of cells in "benign" proliferative lesions (atypical hyperplasia), and in histologically normal breast ductal tissue. Both LOH and microsatellite instability were detected. The abnormalities in normal-appearing tissue were more common in women with cancer than in control (reduction memmoplasty) subjects, and were detected more frequently at chromosomal regions implicated in breast tumorigenesis, compared with randomly selected or neutral sites. These data lead us to hypothesize that certain individuals' breast tissue may contain widespread genetic abnormalities, i.e., "field cancerization". Affected tissue, although normal or benign appearing, would contain a pool of genetically abnormal precursor lesions which could result in increased susceptibility to malignancies, or in malignancies that are distinctive in genetic, clinical or other features.

To investigate these possibilities, we are using our system to compare normal-appearing ductal tissues from several groups of women: those with sporadic cancer vs. those with an hereditary predisposition vs. reduction mammoplasty controls. We are also examining normal-appearing and malignant breast tissues from women exposed prenatally to the potent estrogen compound diethylstilbestrol (DES) vs. controls. We speculate that there will be increased genetic abnormalities in all three groups, compared to controls, and that the pattern of abnormalities may indicate genes or pathways important to the earliest steps of breast tumorigenesis.

b) 4/00:

Clonal Progression of Premalignant Breast Cancer Precursors. Larson PS, de las Morenas A, Rosenberg CL. Boston University Medical Center, Boston MA 02118.

The earliest recognized breast malignancies, carcinomas in situ (CIS), contain multiple abnormalities, suggesting that precursor lesions exist. Hyperplastic lesions are candidate precursors, since epidemiological evidence links them to increased breast cancer risk, and genetic data indicate they can contain clonal abnormalities. However, their relation to malignancies remains unknown. To determine whether ductal hyperplasias are precursors of ductal malignancies, we multiplexed ~20 microsatellites, from 9 chromosomal arms, and examined loss of heterozygosity (LOH) in DNA from multiple lesions microdissected from single specimens. From 14 specimens, 83 controls (stroma, node or epithelium), 9 simple hyperplasias, 38 atypical hyperplasias (AH), 27 CIS and 17 invasive carcinoma (IC) samples were examined. We find 1) in 6/14 subjects (43%) one or more AH shares site(s) of LOH with CIS and/or ICs in the same specimen. Other, histologically identical, AH may not contain the same LOH. 2) LOH seen in histologically normal tissues is not always detected in synchronous hyperplastic or malignant lesions. These data suggest 1) AHs are genetically heterogeneous but at least a fraction are clonally related to cancers, either as direct precursors or by sharing a common precursor. 2) Aberrant clones in normal-appearing tissue are not obligate cancer precursors. These results should help define sequences of genetic abnormalities that result in breast cancer development.

c) 6/00

CLONALITY AND GENETIC INSTABILITY IN PREMALIGNANT BREAST TISSUE

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Multiple genetic abnormalities characterize the earliest recognized breast malignancy, carcinoma in situ (CIS), implying that premalignant precursors exist. Recent data demonstrate hyperplastic lesions, considered benign but associated with increased risk of breast cancer, can contain clonal genetic abnormalites, in particular, loss of heterozygosity (LOH). We hypothesized: 1) LOH might be detectable earlier, perhaps even in histologically normal breast epithelium. If so, aberrant clones might represent very early stages in breast tumorigenesis, and their genetic alterations implicate loci critical to the beginning of cancer development; 2) we could explore breast cancer

progression by examining patterns of LOH in synchronous normal-appearing, hyperplastic and malignant tissues.

To investigate, we used a panel of 10-20 microsatellite markers selected for chromosomal location at known or putative tumor suppressor (ts) genes, % heterozygosity, and size of amplified product, to examine DNA from histologically defined lesions. 95 normal-appearing samples of breast epithelium were microdissected from archived blocks of 6 consecutive cases of reduction mammoplasty, 9 of atypical hyperplasia and 5 of sporadic breast cancer. From these and additional cancer specimens, 83 more normal, 47 hyperplastic, 27 CIS and 17 invasive carcinoma lesions were also microdissected.

We found clonal abnormalities, primarily LOH but occasional microsatellite instability, in 22% (21/95) of histologically normal samples; in women <50 yrs trends towards increased abnormalites were noted with increased breast cancer risk ($p = 0.05$). Abnormalities clustered at sites of known or postulated ts genes vs at more random or neutral sites: 80% (28/35) were at 7q, 11p, 17p, 17q, vs. 20% (7/35) at 1p, 1q, 2p, 18q, Xq ($p = 0.05$). Preliminary investigations into the progression of aberrant clones suggest that multiple independent clones can exist within a single breast, some of which are related, and other unrelated, to the cancer that is present.

Thus, genetic abnormalities are present even in histologically normal breast tissue and genetic instability characterizes certain premalignant breast tissues. Future studies should help distinguish clones that are likely to progress and from those that are not.

American Journal of Pathology, Vol. 152, No. 6, June 1998

Genetically Abnormal Clones in Histologically Normal Breast Tissue

Pamela S. Larson,*† Antonio de las Morenas,† L. Adrienne Cupples,‡ Katie Huang,* and Carol L. Rosenberg*†§

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Breast cancer is believed to develop as multiple genetic abnormalities accumulate, each conferring some growth advantage, but the timing and nature of the earliest steps in this progression are not yet elucidated. Proliferative breast lesions, associated with an increased risk of breast cancer although considered benign, recently were shown to contain clonal genetic abnormalities. Therefore, we hypothesized that clonal genetic abnormalities might be detectable before any phenotypic abnormalities are evident, ie, in histologically normal breast tissue. We examined DNA extracted from 95 normal-appearing breast ducts or terminal ductal-lobular units from 20 individuals at varying degrees of risk (those undergoing reduction mammoplasties, those with atypical hyperplastic proliferative lesions, and those already diagnosed with breast cancer). Using nine microsatellite markers, we sought evidence of genetic instability or of allelic imbalance (most likely representing loss of heterozygosity). We found genetically abnormal clones in 21/95 (22%) seemingly normal samples from 10/20 (50%) women from all three risk groups. In women under age 50, trends toward increased rates of abnormalities were noted with increased cancer risk. The abnormalities identified were more likely to be at sites of known or postulated tumor suppressor genes rather than at random or neutral loci. Our data indicate that genetic abnormalities potentially critical to breast tumorigenesis accumulate before pathological detection even of high-risk lesions and are detectable in tissue that is not only histologically benign but also completely normal. ***(Am J Pathol 1998, 152:1591–1598)***

Breast cancer is believed to develop as multiple genetic abnormalities accumulate, each conferring some growth advantage. Aberrations of oncogenes, loss of genetic material, and some degree of genomic instability can be detected in breast cancers of all stages.[1–9] Recent evidence indicates that at least a subset of proliferative breast lesions may also be characterized by clonal genetic aberrations, including loss of heterozygosity (LOH) or some type of microsatellite instability.[10–17] Histologically, these lesions are considered to be benign, although epidemiologically they are associated with increased risk of cancer development. Thus, some of these lesions could represent actual precursors of malignancy. Based on these findings, we hypothesized that some genetic abnormalities may have occurred even earlier, ie, before the development of histologically abnormal tissue and, therefore, might be detectable in normal-appearing breast ductal tissue. Finding genetic abnormalities in histologically normal breast tissue would imply that genetically abnormal clones develop and can be identified much earlier than has been appreciated. In addition, the nature of any identified abnormalities could indicate events important to the initial steps of breast tumorigenesis.

To investigate this hypothesis, we examined multiple samples of histologically normal breast ductal tissue from 20 individuals' archival specimens. Both single ducts and the larger terminal ductal-lobular units (TDLUs) were examined, as it was recently demonstrated that TDLUs are likely to represent the progeny of a single breast ductal precursor or stem cell.[18, 19] Although this was a pilot study examining specimens from 20 individuals, we selected breast tissue from three distinct groups of subjects: 1) those at no increased risk of breast cancer (reduction mammoplasties), 2) those without a history of breast cancer but who are at a four- to five-fold increased risk of developing the disease because of a biopsy revealing an atypical hyperplastic (AH) lesion, and 3) those already diagnosed with breast cancer (lumpectomies and mastectomies).

Each sample of normal tissue was examined with a panel of nine highly informative microsatellite markers. The markers were selected so that approximately one-half were situated at chromosomal regions known to be lost or mutated in breast cancer (and therefore might represent sites of tumor suppressor genes important to breast tumorigenesis); the other half were at genes or anonymous sequences not known to be relevant to breast tumorigenesis. Additionally, loci were selected to

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achieve a mixture of di-, tri-, and tetranucleotide repeats. In most breast cancers, the microsatellite instability seen is not the widespread dinucleotide repeat alterations characteristic of tumors lacking normally functioning mismatch repair genes. Instead, changes are seen in more often in tri- or tetranucletide repeats, and at comparatively lower frequency, suggesting a more subtle defect maintaining genomic integrity.[3, 8]

Using these specimens and this panel of markers, we determined the incidence and pattern of genetic abnormalities in multiple independent histologically normal samples of breast ductal tissue.

Materials and Methods

Selection of Samples

All samples were reviewed by a single breast pathologist to identify histologically normal tissue. In total, 95 samples of histologically normal single ducts or TDLUs were obtained from 20 subjects; an average of 5 ducts or TDLUs were examined per subject. In addition, lymph node and stromal tissues were examined when available. Six cases of reduction mammoplasties were identified at random from the pathology department archives; candidates were screened before surgery to eliminate those in whom there is a personal or familial history of breast cancer. Nine cases of AH had been identified previously.[14] Five cases of breast cancers diagnosed in pre- or perimenopausal women were selected at random from the pathology department archive. Breast cancer cases were selected in an attempt to match the uniformly young age of the reduction mammoplasty subjects and the relatively young age of those with AH. Most specimens dated from 1994 or 1995; a few were older. Initial diagnostic or therapeutic specimens were used, and therefore, no subjects had received prior chemotherapy or radiation. Tissue from both right and left sides was identified in all reduction mammoplasty and in breast cancer subject 34, who had bilateral disease. Except in this case, no subject with breast cancer was aware of a positive family history. Tissue from only the affected side was available in the remaining cases.

Preparation of DNA

Histologically normal single ducts or TDLUs, stromal tissue and lymph nodes, were identified by hematoxilyn and eosin (H&E) staining of the top and bottom of seven consecutive sections cut from a tissue block; the five intervening unstained sections were then microdissected as previously described.[14] DNA was extracted using standard techniques.[20, 21] To make sure abnormalities were not artifacts due to small amounts of template DNA,[22] we performed serial dilution experiments. Using normal lymphocyte DNA, we determined the concentration at which the pattern of the products amplified by the AR primers was no longer reproducible. In each case, the amplified products were identical in size and intensity when 100 pg or more of template DNA was used, but with 10 pg, the results were inconsistent (data not shown). To approximate the minimal DNA concentration in each reaction using DNA from ducts or TDLUs, we determined that the number of cells microdissected from a normal duct varied between 250 and 1000; a TDLU contains far more cells. Assuming 6.5 pg of DNA per cell and 50% loss of DNA during extraction, and given that 1/10 of the final volume of DNA solution was used per reaction, we estimate that a minimum of between 80 and 325 pg of template DNA was available per reaction.

Microsatellites

After DNA extraction, each sample was examined using nine microsatellite markers at nine genomic loci. The nine microsatellite sequences examined were MYCL1 (1p), D1S549 (1q), D2S123 (2p), D7S486 (7q), THO1 (11p), TP53 (17p), D17S579 (17q), D18S34 (18q), and AR (Xq). Primers were purchased from Research Genetics (Huntsville, AL) or synthesized commercially.

Polymerase Chain Reaction and Data Analysis

We performed multiplex polymerase chain reactions (PCRs) as described elsewhere.[14] Briefly, 1/10 of the DNA solution served as a template in a 50-μl reaction volume. After 40 cycles of amplification incorporating [α^{32}P]dCTP, with annealing temperatures of 55°C, 58°C, or 60°C, one-fifth of the amplified products were electrophoresed through 7% denaturing gels. Microsatellite changes were scored by visual inspection as instability (when a novel-sized amplified product was present) or as allelic imbalance suggestive of LOH when unequivocal loss of intensity of one allele was seen at heterozygous loci. To be scored as abnormal, demonstration of instability or LOH needed to be reproduced at least twice with identical results. Because of the limited quantities of DNA available, unequal amplification in early PCR cycles could lead to inaccurate relative allele intensities; therefore, ratios of relative allele intensities at heterozygous loci based on densitometry were not calculated, and relative allele imbalance was scored as no loss. After scoring, the total number of abnormal samples and of abnormal alleles, and the nature of the abnormalities, were determined.

Statistical Analysis

We compared the three groups on the following measures: 1) the proportion of subjects with at least one abnormality, 2) the mean percentage of abnormal loci, and 3) the mean percentage of abnormal ducts. The data were examined for all subjects and, separately for women under age 50, by analysis of variance (weighting by the number of ducts or TDLUs).

Results

Histologically normal breast ducts and TDLUs (samples) were microdissected carefully (see Figure 1). We de-

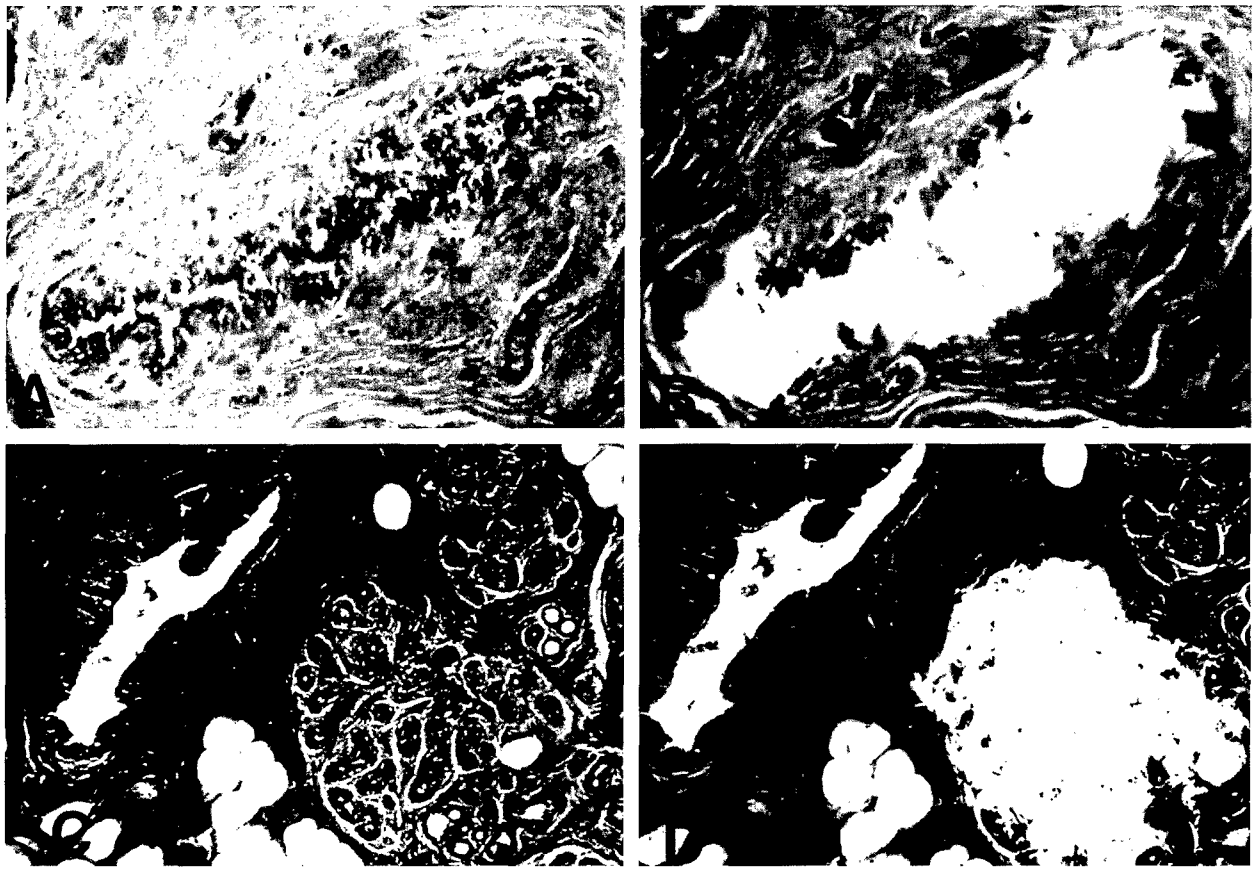

Figure 1. Examples of tissue before and after microdissection. Slides were stained with H&E for the purpose of illustration. Subject 35's sample 6 (duct) is shown before (A) and after (B) microdissection. Magnification, ×200. Subject 35's sample 2 (TDLU) is shown before (C) and after (D) microdissection. Magnification, ×100. Amplification of the DNA extracted from these two samples is shown in Figure 3D.

tected genetically abnormal clones of cells in these normal-appearing samples in 10/20 (50%) subjects studied; 21/95 (22%) ducts or TDLUs were involved. The data are presented in Table 1. Alterations were present in subjects from all three groups, ie, in 2/6 (33%) women without apparent increased risk of breast cancer (reduction mammoplasty), in 4/9 (44%) women with an increased risk of breast cancer (AH), and in 4/5 (80%) women who already had developed the disease. More than one independent abnormal clone was detected in 1/6 (17%) subjects who had undergone reduction mammoplasty (subject 32), in 1/9 (11%) subjects with AH (subject 19), and in 4/5 (80%) subjects (subjects 34, 35, 36, and 37) with breast cancer.

Samples characterized by microsatellite changes were histologically indistinguishable from those without (see Figure 2), and no preference for abnormalities in single ducts compared with TDLUs was seen. Somewhat more microsatellite instability (22 examples) than LOH (13 examples) was evident, but this may have been skewed by one subject (12) with nine instances of instability and only one of LOH. In addition, because relative allele imbalance was scored as no loss (due to the small quantity of template DNA; see Materials and Methods), it is possible that we underestimated the number of cases characterized by LOH. However, as each duct or TDLU likely represents the progeny of a single ductal stem cell,[18] allelic alterations should usually affect at least a substantial fraction of the cells comprising the sample and therefore should, generally, be detectable.

From six subjects undergoing reduction mammoplasties, who had no increased risk of breast cancer, 32 histologically normal samples were examined. Five ducts from two individuals contained clonal genetic changes. Subject 24 had a single abnormality identified in one of five samples (data not shown). In subject 32 (see Figure 3A), 4 of 10 samples, all from the right breast, had evidence of five microsatellite abnormalities, four of which involved a single microsatellite locus. In this subject, more than one abnormal clone was present. To determine with certainty this subject's germline configuration at these loci, three geographically distinct samples of stromal tissue were microdissected from the same blocks. All three demonstrate a single pattern, identical to that seen in the majority of the ducts (see Figure 3A).

From nine subjects diagnosed with high-risk AH lesions (some of which have been shown to contain clonal abnormalities[14]), a total of 26 histologically normal samples were examined. Because the diagnosis of AH is usually made from a biopsy, the amount of tissue available for investigation is much smaller than from reduction mammoplasty or cancer specimens, and it is always unilateral. Despite the smaller number of samples exam-

Table 1. Microsatellite Alterations in Histologically Normal Breast Tissue Samples

Subject	Age	Number of microsatellite loci	Number of altered samples/total samples	Number of altered alleles/total alleles examined*	Type of alteration
Histologically normal ducts or TDLUs from reduction mammoplasty specimens (n = 6)					
24	34	9	1/5	1/90	1 instability
25	25	9	0/4	0/72	
26	24	9	0/3	0/54	
29	42	9	0/3	0/48	
31	23	7	0/7	0/92	
32	36	8	4/10	5/138	1 instability, 4 LOH
Subtotal		9	5/32 (15.6%)	6/494 (1.2%)	2 instability, 4 LOH
Histologically normal ducts or TDLUs from AH biopsies (n = 9)					
10	58	9	0/3	0/50	
12	63	8	1/6	10/92	9 instability, 1 LOH†
13	37	8	0/2	0/32	
17	51	8	0/3	0/48	
18	31	8	0/2	0/32	
19	41	8	2/2	2/32	2 instability
20	74	7	1/3	2/40	2 LOH
21	59	5	1/3	1/26	1 LOH
23	63	8	0/2	0/32	
Subtotal		8	5/26 (19.2%)	15/384 (3.9%)	11 instability, 4 LOH
Histologically normal ducts or TDLUs from subjects with breast cancer (n = 5)					
34	39	7	3/7	3/84	1 instability, 2 LOH
35	38	7	2/11	4/154	4 instability†
36	39	7	3/8	3/98	2 instability, 1 LOH
37	38	8	3/6	4/74	2 instability, 2 LOH†
38	53	6	0/5	0/60	
Subtotal		7	11/37 (29.7%)	14/470 (3.0%)	9 instability, 5 LOH
Total		~8	21/95 (22.1%)	35/1348 (2.6%)	22 instability, 13 LOH

*Occasional amplifications either were not successful or did not yield reproducible results; in these cases, the alleles were not scored, and hence, the actual number of alleles examined is in some instances slightly smaller than the maximum possible number would be. The maximum possible number of evaluable alleles equals: (number of primers) × (number of ducts) × (two alleles per locus).

†One or more biallelic changes noted.

ined per subject in this group than in either of the others, we identified evidence of microsatellite alterations in 5 of 26 histologically normal breast samples from 4 of 9 subjects in this high risk group (subjects 12, 19, 20, and 21). Subject 12 demonstrated 10 microsatellite alterations, all present in one of six samples (see Figure 3B). (This subject had colon cancer diagnosed at age 60, 3 years before breast biopsy, and 9 years later remains free of disease. It is possible that she represents a case of hereditary nonpolyposis colon carcinoma). Subject 19 demonstrated microsatellite instability in each of the two samples examined. A different locus was altered in each sample; thus, this subject had more than one abnormal clone. In subjects 20 and 21, one of three samples was abnormal, each with evidence of LOH (data not shown). Interestingly, the AH lesions from these four subjects were not found to have allelic alterations.[14] Similar dissonance between genetic abnormalities in AH and in simple hyperplastic lesions from the same subject has been reported recently.[17]

The third group studied consisted of five subjects with breast cancer, from whom a total of 37 histologically normal ducts and TDLUs were microdissected. Seemingly normal tissues from four of five subjects contained genetic abnormalities. In all four subjects, multiple abnormal clones were found. Three of seven samples from subject 34 contained genetic alterations at three different loci (see Figure 3C). Samples from both left and right breast were abnormal. As a control to confirm the subject's germline configuration at the apparently altered loci, DNAs from three lymph nodes pathologically free of tumor were examined. Their microsatellite patterns were the same as the predominant pattern seen in the ducts or TDLUs (see Figure 3C). Two of eleven samples from subject 35 contained four genetic alterations involving two microsatellite loci (see Figure 3D). As a control, lymphoid tissue from three separate nodes was examined; no microsatellite alterations were detected. Three of eight samples from subject 36 demonstrated three genetic alterations at three separate loci (see Figure 3E). Finally,

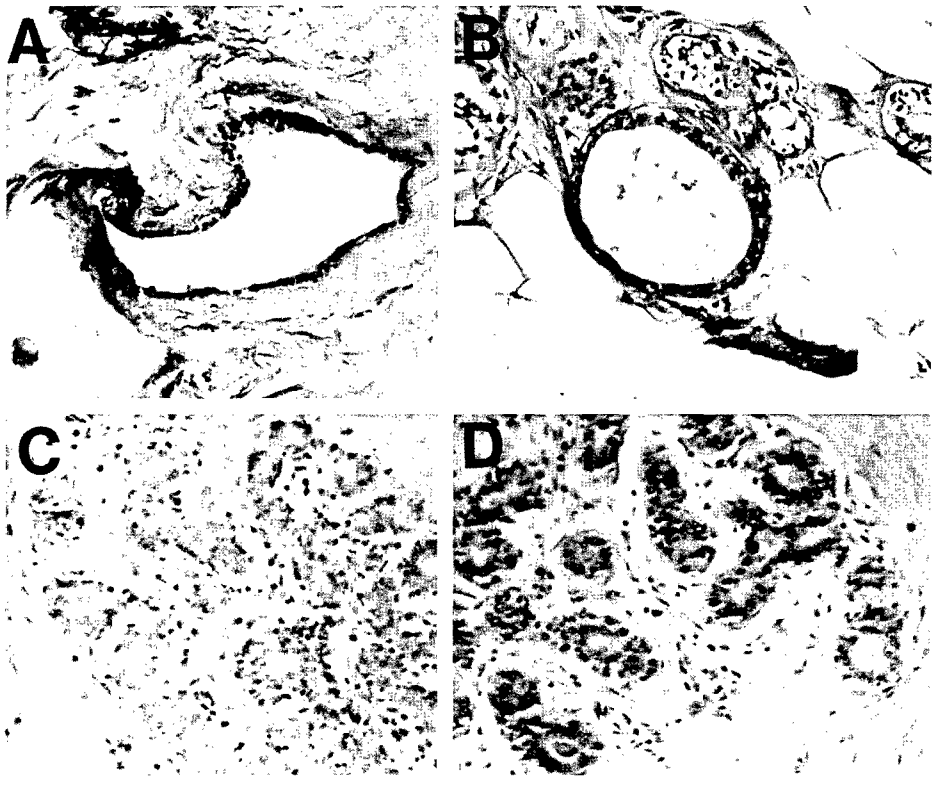

Figure 2. Normal-appearing ducts and TDLUs. Those with or without genetic alterations are histologically indistinguishable. A: From subject 12, sample 6, a genetically normal duct. B: Sample 3, which is genetically aberrant. C: From subject 32, sample 3, a genetically normal TDLU. D: Sample 2, which is genetically aberrant. Magnification, ×100.

three of six samples from subject 37 revealed four abnormalities at three microsatellite loci (data not shown). The remaining subject (38), with five samples examined, demonstrated no abnormalities.

Overall, a total of 35 clonal alterations were detected among 1348 alleles examined, yielding a mutation rate of 2.6%. There were suggestions that as the risk of breast cancer increased so did the number of alterations, particularly for women less than 50 years old. For example, the percentage of all subjects with any abnormality increased from 33.3% to 44.4% to 80.0% across the three groups, and the mean percentage of abnormal alleles rose from 1.2% in women with reduction mammoplasties to 3.9% in women with AH and to 3.0% in women with breast cancer. Similarly, the mean percentage of abnormal ducts increased with risk of breast cancer, from 15.6% in the reduction mammoplasty group to 19.2% in the AH group and to 29.7% in the breast cancer group. However, perhaps due to the relatively small sample size of this pilot study, these observations did not achieve statistical significance. When women under age 50 were examined, the trends were more pronounced and reached significance when comparing mean percentage of abnormalities between the subjects with reduction mammoplasty (1.2%) and those with breast cancer (3.4%; $P = 0.049$; see Table 2).

Certain microsatellite loci were altered much more commonly than others. Four of the nine loci examined accounted for 28/35 (80%) abnormalities. In contrast, the remaining five loci accounted for only one-fourth as many: 7/35 (20%) abnormalities. When LOH alone was considered, the results were similarly skewed; 11/13 examples of LOH were at these four loci, whereas only 2/13 examples of LOH were at the other five loci. The four frequently altered loci are all situated near sites of known or putative tumor suppressor genes postulated to be relevant to breast tumorigenesis: 7q31,[23] 11p15,[24, 25] 17p13,[26] and 17q21.[27, 28] In contrast, the five less frequently altered loci are situated at genes or sites less commonly associated with breast cancer. When microsatellite instability was considered, we found that 12/22 abnormalities were seen at the five dinucleotide repeat markers and 10/22 at the four tri- and tetranucleotide repeat markers. This pattern reflects that reported in breast cancer.[3, 8, 9]

Discussion

We have found multiple genetically abnormal clones existing in breast tissue, although that tissue looks not only benign but also histologically completely normal. These data indicate that genetic abnormalities that may be critical to breast tumorigenesis start accumulating far before pathological detection even of high-risk lesions. The eventual fate of a given clone is unknown, as is the risk to a woman whose breast contains these occult lesions. It is noteworthy that we find mutant clones both in women at low and at high risk of developing breast cancer, because the majority of women who develop breast cancer

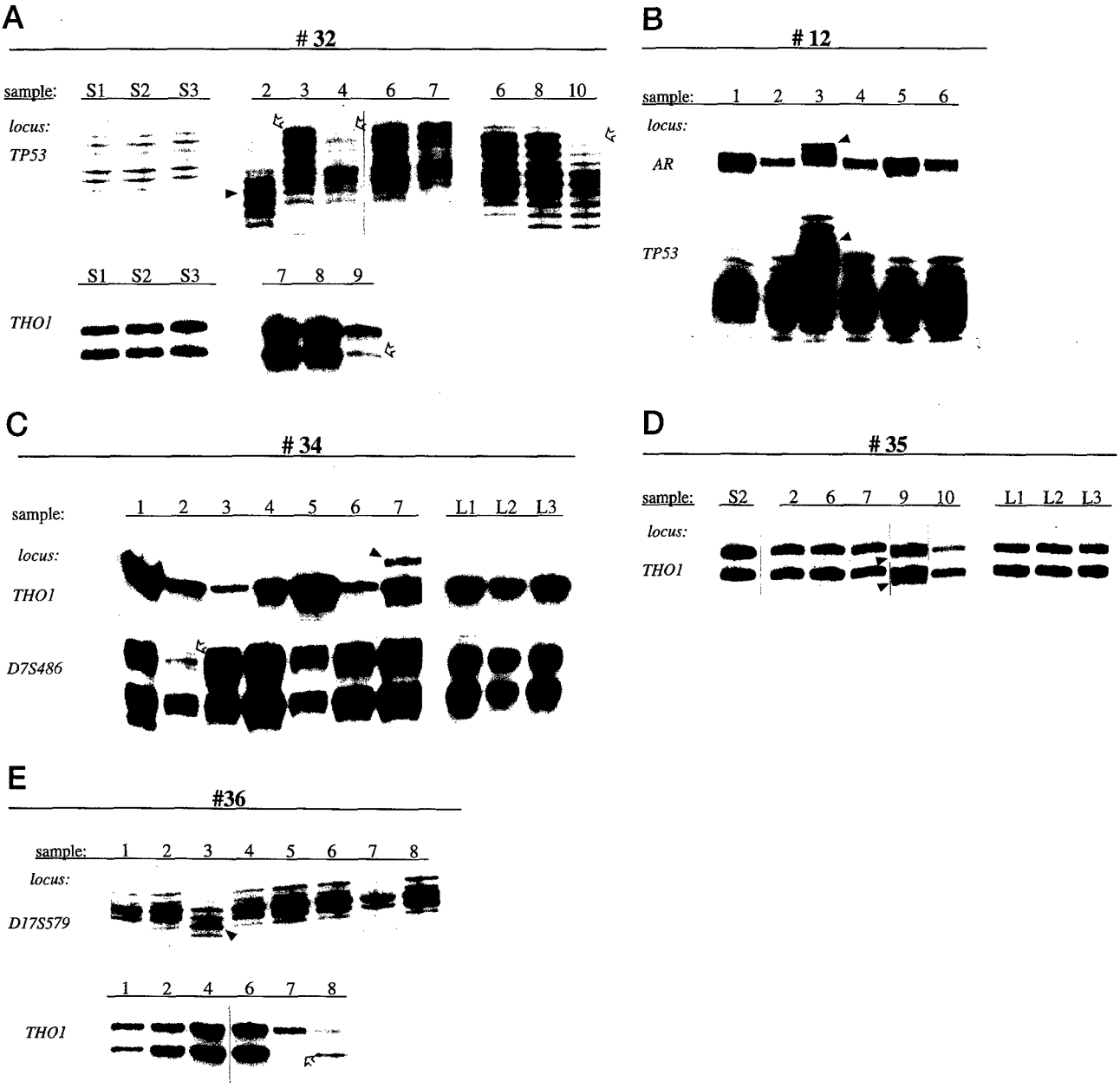

Figure 3. Representative examples of genetic abnormalities seen in histologically normal ducts or TDLUs from five subjects. A: LOH and microsatellite alteration at two loci in subject 32, who had a reduction mammoplasty. LOH at the upper allele of the TP53 microsatellite is seen in samples 2, 4, and 10 (open arrows). In addition, in duct 2, the lower TP53 allele is smaller than normal (closed arrow). The bottom part of the panel demonstrates LOH at the lower THO1 allele in duct 9 (open arrow). S1, S2, and S3 are three separate stromal specimens, each demonstrating the same unaltered pattern at both loci. B: Microsatellite alterations at two loci in subject 12, diagnosed with AH. A larger band, representing a novel allele, is seen at both the AR and TP53 microsatellites in sample 3 (closed arrows) but in none of the other five ducts. C: LOH and microsatellite alteration at two loci in subject 34, with breast cancer. A larger band, representing a novel allele, is seen in sample 7 at the THO1 microsatellite (closed arrow), and LOH of the upper allele at the D7S486 microsatellite is seen in sample 2 (open arrow). L1, L2, and L3 are three separate lymph nodes, each demonstrating the same pattern of microsatellite bands, representing the germline pattern. D: Biallelic alterations (closed arrows) at the THO1 locus in sample 9 from subject 35. One sample of stromal tissue (S2) and three lymph nodes (L1, L2, and L3) were also examined and demonstrated no alterations. (Photographs of samples 2 and 6 before and after microdissection are shown in Figure 1). All ductal samples were amplified and electrophoresed simultaneously, but different exposures have been placed adjacently. E: LOH and microsatellite alteration at two loci in subject 36, with breast cancer. At the D17S579 microsatellite, a shortened allele replacing the upper allele is seen in duct 3 (closed arrow). At the THO1 microsatellite, LOH of the lower allele is seen in duct 7.

have no identifiable risk factors. Clinical follow-up of the individuals in this study is not currently available.

It is possible that the mutant clones we detect are relevant to the earliest stages of breast tumorigenesis. Several observations support the speculation that these clones may indicate tissue at increased risk of cancer development. First, 4 of 5 women with breast cancer had multiple abnormal clones, whereas only 2 of 15 women without the disease had more than a single abnormal clone. Second, 80% of the microsatellite abnormalities were at four loci believed to play a role in breast tumorigenesis. LOH, suggesting the presence of a tumor suppressor gene, has been found at 7q31[23] in a subset of breast cancers; the recently identified *TSG101* putative

Table 2. Rates of Genetic Abnormalities in Normal-Appearing Breast Tissue from Subjects <50 Years Old in Three Breast Cancer Risk Groups

Group	% subjects with abnormality	Mean % abnormal alleles	Mean % abnormal ducts
Mammoplasty	33.3	1.2	15.6
AH	33.3	2.1	33.3
Breast cancer	100.0*	3.4†	34.4‡

*P = 0.076 *versus* mammoplasty group.
†P = 0.049 *versus* mammoplasty group.
‡P = 0.123 *versus* mammoplasty group.

tumor suppressor gene, located at 11p15,[24, 25] is mutated in a fraction of human breast tumors; mutations of the *P53* tumor suppressor gene, located at 17p13,[26] are the most frequently identified genetic abnormalities in breast cancer; and the breast cancer susceptibility gene, *BRCA1*, and possibly other relevant tumor suppressor genes, are found at 17q21.[27, 28] Although *BRCA1* itself has not been found to be mutated in a significant percentage of sporadic human breast cancers,[29] LOH in the region of the gene is detectable.[27, 28] Thus, another mechanism of *BRCA1* inactivation or another gene may be playing a role.

Although these microsatellite loci were selected because of their chromosomal location, the overrepresentation of abnormalities at these sites indicates that they may predispose to the formation of genetically aberrant clonal populations. In contrast, mutation at arbitrary or more neutral sites may not confer a growth advantage, and a detectable mutant clone may never arise. This would suggest that the genetic alterations we have detected are less likely to be random changes and more likely to be relevant to the earliest stages of breast cancer development. Finally, it is noteworthy that the pattern of microsatellite instability seen in normal-appearing tissues is similar to the type of instability reported in breast cancers, ie, overall, a low level of microsatellite alterations, with a substantial proportion of changes seen in tri- and tetranucleotide repeat markers.[3] Microsatellite instability has been detected in all stages of breast cancer, and consequently, it has been postulated that this abnormality occurs early in the course of disease development.[2, 4, 6–9]

Our findings in subjects with breast cancer are consistent with the limited data available from studies in other tissues indicating that histologically normal tissue at increased risk for the development of cancer can contain specific clonal genetic abnormalities. For example, clones of p53 mutated keratinocytes occur in sun-exposed normal-appearing human skin[30, 31] and in normal-appearing mucosa from patients with cancers of the upper aerodigestive tract.[32] Microsatellite alterations have been seen in normal-appearing colonic mucosal epithelium of patients with chronic ulcerative colitis, who are at increased risk of developing colon cancer.[33] Finally, LOH at chromosome 3p has been reported recently in breast cancers and in directly adjacent, but not more distant, histologically normal breast tissue.[19] In contrast, cytogenetic studies examining macroscopically normal breast tissue surrounding breast cancers[34] and investigations of LOH and/or microsatellite instability in breast cancers have not reported abnormalities in normal-appearing tissues.[1–9] This may be due, in part, to the relatively large amount of normal tissue generally used as a control, making detection of small abnormal clones difficult.

Even if only a rare abnormal clone expands by acquiring additional mutations and the others represent dead ends (ie, they would involute or remain stable), these data could help explain the genetic heterogeneity noted in many breast cancers. Multifocal breast cancers can represent independent, not metastatic, malignancies,[35] single breast malignancies can contain karyotypically unrelated clones,[36, 37] and heterogeneous patterns of allelic loss have been reported in ductal carcinoma *in situ* tumors.[38] It is unclear how all the distinct clones could represent outgrowths from a single original population. The presence of multiple genetically distinct abnormal clones, several of which could progress independently and simultaneously, could represent one explanation.

Finally, it is notable that a trend may exist in the rate of abnormalities among the three groups of women studied, particularly when in women <50 years of age. Several factors may explain the absence of statistical significance associated with most of these associations. First, the baseline rate for somatic mutation in normal breast tissue may be relatively high, even in women at no identifiable increased risk of breast cancer. We obtained a rate of 1.2% in women with reduction mammoplasties and an overall rate of 2.6%, both of which are higher than the baseline rate of somatic mutation estimated to be <0.5% in clones derived from normal T cells.[39] The rate is suspected to be low in other normal tissue but, as far as we are aware, has not been measured. In addition, the data from the reduction mammoplasty group could have been skewed by the presence of subject 32; this individual had quite a few clonal abnormalities. One could speculate about whether women with abnormalities similar to those of subject 32 could be at higher risk for the eventual development of breast cancer. Second, although the number of alleles examined was large, the number of individuals in each of the three groups may have been too small to detect small but significant differences. Examination of additional specimens from more subjects could clarify this important point. Third, the rate of abnormalities seen in each group may not be related to risk but may reflect the effects of aging. The average age was lowest in the reduction mammoplasty group (31 years) with the lowest rate of abnormalities, intermediate in the breast cancer group (41 years) with the highest rate of abnormalities, and highest in the AH group (53 years) with an intermediate rate of abnormalities; but in this last group, more abnormalities were seen in specimens from older women. Examination of specimens from a larger group of women of varied ages may answer this question. Finally, it is possible that the critical event is mutation of a postulated breast-tissue-specific gatekeeper gene, without which progression of any nascent clone does not occur.[40] Thus, the observed mutation rate might not be the key factor.

Acknowledgments

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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 18 November 2002

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant DAMD17-97-1-7191. Request the limited distribution statement for Accession Document Numbers ADB262448, ADB265603 and ADB282072 be changed to "Approved for public release; distribution unlimited." These reporst should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management